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(54) Title: METHOD FOR IDENTIFYING CHEMICAL ACTIVE AGENTS AND ACTIVE AGENTS FOR INHIBITING THE 1-DESOXY-D-XYLULOSE-5-PHOSPHATE BIOSYNTHETIC PATHWAY

(54) Bezeichnung: VERFAHREN ZUR IDENTIFIZIERUNG CHEMISCHER WIRKSTOFFE UND WIRKSTOFFE ZUR HEMMUNG DES 1-DESOXY-D-XYLULOSE-5-PHOSPHAT-BIOSYNTHESEWEGS

(57) Abstract

The invention relates to a method for identifying chemical active agents which are suitable for treating infectious diseases caused by single- or multi-celled parasites. According to the method, proteins which form part of the 1-desoxy-d-xylulose-5-phosphate metabolic pathway or derivatives thereof which act in the same way are brought into contact with the active agents being tested for their effectiveness against parasites and those active agents which inhibit the proteins or their derivatives are selected. The invention also relates to the active agents which are identified and to their use for producing medicaments for treating parasitic infections.

(57) Zusammenfassung

Die Erfindung betrifft ein Verfahren zum Auffinden von chemischen Wirkstoffen, die zur Therapie von Infektionskrankheiten geeignet sind, die durch ein- oder mehrzellige Parasiten hervorgerufen werden. Bei diesem Verfahren werden Proteine, die am 1-Desoxy-D-xylulose-5-Phosphat-Stoffwechselweg beteiligt sind, oder deren gleichwirkende Derivate mit den auf ihre Wirksamkeit gegenüber Parasiten zu untersuchenden Wirkstoffen in Berührung gebracht und die Wirkstoffe, die die Proteine oder deren Derivate inhibieren, ausgewählt. Die Erfindung betrifft ferner die aufgefundenen Wirkstoffe zur Herstellung von Arzneimitteln gegen parasitäre Infektionen.

Process for identifying chemical active ingredients and active ingredients for inhibiting the 1-desoxy-D-xylulose-5-phosphate biosynthesis pathway

The invention relates to a process for identifying active ingredients which are suitable for treating parasitic diseases caused by unicellular or multicellular parasites. Medicine and the pharmaceutical industry are the areas of application of the invention. The invention also relates to proteins, and fragments of proteins, also DNA-sequences which code these proteins or fragments of proteins, the use of these DNA-sequences, these proteins or their fragments for identifying substances acting against unicellular or multicellular parasites, and the active ingredients identified in this way and their use for producing pharmaceutical compositions.

The term parasites includes unicellular parasites and multicellular parasites including Helminthes and anthropoidea. These cause infectious diseases in humans and animals. In the context of this invention, the strictly scientific definition of parasites is to be used, i.e. unicellular parasites are to be take to mean Protozoa.

A large number of preparations against parasitic diseases already exist. The preparations available are already becoming useless for treating humans and animals due to fast developing resistance. As a result there are already many regions affected by malaria parasites which

are resistant to standard medications such as chloroquine. Reports are also known about development of resistance to standard preparations (praziquantel) for treating bilharziosis. These developments of resistance and other factors have led to the fact that malaria and bilharziosis are already amongst the most frequent diseases in the Tropics. An estimated 300-500 million people are suffering from malaria. 2-2.5 million people die annually of malaria. New medications such a mefloquin are, furthermore, very expensive to produce and have many side effects. There is therefore a great need for pharmaceutical compositions for treating humans and animals.

In the past there were many attempts at developing chemotherapy compositions against parasites, in particular against malaria and bilharziosis pathogens. One of these attempts is concerned with inhibiting so-called isoprenoid biosynthesis. Isoprenoids are molecules which are formed from individual isoprene units (isopentenyldiphosphate) and adopt important functions in the cell. These include sterols, ubiquinones and other molecules which are important for the parasite household. The process of proceeding was based, in this case, on a model which was established in fungi and mammalian cells. In fungi and in mammalian cells the sub-unit isopentenyldiphosphate is formed by condensation of three acetyl-CoA molecules to HMG-CoA. HMG-CoA is then converted from the HMG-CoA-reductase to mevalonate which is then converted with mevalonate phosphate as an intermediate stage to

isopentenyldiphosphate (see Fig. 7). HMG-CoA-reductase inhibitors such as, for example Lovastatin, Simvastatin and Pravastatin have been used for inhibiting the growth of parasites. Although it was possible to obtain in vitro inhibition by using very high doses of Lovastatin and Simvastatin, in vivo inhibition failed. Treatment of Schistosome-infected mice with Lovastatin led to inhibition of the egg-laying by these worms, however, very high concentrations of Lovastatin had to be used to destroy some of these worms in vivo.

Surprisingly it has been found that parasites, in particular plasmodia and trypanosomes (causes of malaria and sleeping sickness) have at least one further metabolic pathway to the synthesis of isoprenoids. This metabolic pathway is based on condensation of glyceraldehyde-3-phosphate and pyruvate to 1-desoxy-D-xylulose-5-phosphate (DOXP). DOXP is then converted to 2-C-methyl-D-erythrose-4-phosphate which is then converted to 2-C-methyl-erythrithol-4-phosphate as intermediate stage to isopentenyldiphosphate. The enzymes DOXP-synthase and DOXP-reductoisomerase *inter alia* are involved in this metabolic pathway (see Fig. 7). In the past this metabolic pathway had only been described in plants, in algae and in some bacteria (Sprenger et al. PNAS, 94 (1997) 12857-62 und Kuzuyama et al. Tetrahedron Letters 39 (1998) 4509-12).

Inhibition of the DOXP-metabolic pathway described above, in particular the enzymes DOXP-synthase and DOXP-reductoisomerase, by techniques known to the person

skilled in the art is suitable for preventing and treating infections caused by unicellular and multicellular parasites in humans and animals. As this metabolic pathway does not occur in humans, it is ideally suited as the target for a selective chemotherapy of parasites. The enzymes desoxyxylulose-5-phosphate-synthase and desoxyxylulose-5-phosphate-reductoisomerase are particularly suited as the target for chemotherapy. The inhibition of the enzyme desoxyxylulose-5-phosphate-reductoisomerase of malaria proved to be particularly low in side effects and suitable, as man does not have substrates and their precursors nor the product of the enzyme nor the enzyme itself.

The present invention relates to processes for obtaining active ingredients which inhibit the DOXP-metabolic pathway and to these active ingredients for producing pharmaceutical compositions for the treatment and prophylaxis of infectious diseases caused by unicellular or multicellular parasites.

It is the object of the invention to provide a new process for identifying active ingredients for the treatment of parasitic diseases in humans and animals. A further object is to develop a process for obtaining a medication which selectively destroys the pathogen and has few side effects.

This object is achieved by a process according to claim 1. The process according to the invention and the active ingredients found are characterised in that

- isoprenoid biosynthesis in the so-called 1-desoxy-D-xylulose-5-phosphate metabolic pathway is inhibited.

None of the metabolic pathways described are present in humans and animals, only in plants, algae, some eubacteria and in parasites such as, for example, malaria parasites; this treatment strategy stands out therefore as having few side effects.

The present invention further relates to enzymes involved in this metabolic pathway and fragments of these enzymes. These enzymes are proteins suitable for carrying out the process according to the invention for identifying active ingredients. The present invention further relates to DNA-sequences which code these enzymes or fragments of these enzymes.

The present invention relates to a process and antibodies for identifying the enzymes or their fragments and producing the enzymes or their fragments by means of recombinant technology.

The invention further relates to the use of these enzymes or their fragments or the use of the DNA-sequences which code these enzymes or fragments of these enzymes for identifying substances active against unicellular or multicellular pathogens.

The invention further relates to active ingredients discovered with the aid of the enzymes according to the invention.

The invention will be described in more detail hereinafter with the aid of the accompanying drawings, in which:

Fig. 1a shows the nucleotide sequence of the gene coding the protein 1-desoxy-D-xylulose-5-phosphate-reductoisomerase from Plasmodium falciparum,

Fig. 1b shows the nucleotide sequence of the gene coding the 1-desoxy-D-xylulose-5-phosphate-synthase from Plasmodium falciparum,

Fig. 2a shows the nucleotide sequence of the gene coding the 1-desoxy-D-xylulose-5-phosphate-reductoisomerase from Plasmodium falciparum and the corresponding amino acid sequence,

Fig. 2b shows the nucleotide sequence of the gene coding the 1-desoxy-D-xylulose-5-phosphate-synthase from Plasmodium falciparum and the corresponding amino acid sequence,

Fig. 3a shows the amino acid sequence of the protein 1-desoxy-D-xylulose-5-phosphate-reductoisomerase from Plasmodium falciparum,

Fig. 3b shows the amino acid sequence of the protein 1-desoxy-D-xylulose-5-phosphate-synthase from the parasites Plasmodium falciparum,

Fig. 4a is a detail from the nucleotide sequence according to Fig. 1b,

Fig. 4b is a detail from the nucleotide sequence with the corresponding amino acid sequence according to Fig. 2b,

Fig. 4c is a detail from the amino acid sequence according to Fig. 3b,

Fig. 5 shows in vivo data for the parasitemia values after 4 days of treatment with 3 doses in each case of the substances:
formyl, corresponding to 3-(N-formyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt, and
acetyl, corresponding to 3-(N-acetyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt,

Fig. 6a shows the inhibition of the growth of P. falciparum after addition of 3-(N-formyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt (open circles) and 3-(N-acetyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt (closed circles) for the HB3 strain,

Fig. 6b shows the inhibition of the growth of P. falciparum after addition of 3-(N-formyl-N-

hydroxylamino)-propyl-phosphonic acid monosodium salt (open circles) and 3-(N-acetyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt (closed circles) for the A2 strain and

Fig. 6c shows the inhibition of the growth of P. falciparum after addition of 3-(N-formyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt (open circles) and 3-(N-acetyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt (closed circles) for the Dd2 strain, and

Fig. 7 shows the classic acetate/mevalonate biosynthesis pathway in comparison to the alternative DOX-P-biosynthesis pathway.

The coding genes of the enzymes DOXP-synthase and DOXP-reductoisomerase were detected by genetic processes (Fig. 1a, 1b, 2a, 2b). After enrichment by the polymerase chain conversion from the genome of P. falciparum these genes were cloned in bacterial plasmids and their nucleotide sequence determined. The sequence data showed a high homology of these genes with the corresponding genes from algae, plants and bacteria. The very high homologies showed that the three genes code the enzymes DOXP-synthase and DOXP-reductoisomerase of P. falciparum.

After expression in heterologous systems the enzymes were purified as recombinant proteins and used for activity studies in cell-free systems. The activity of

the DOXP-synthase was measured by converting glyceraldehyde-3-phosphate and pyruvate to 1-desoxy-D-xylulose-5-phosphate. The activity of the DOXP-reductoisomerase was measured by converting 1-desoxy-D-xylulose-5-phosphate to 2-C-methyl-D-erythritol-4-phosphate in the presence of NADPH. Measurement of the change in the NADPH concentration is via a parameter variation. This process is known to the person skilled in the art.

The enzymes can be defined by the DNA-sequence coding them (Fig. 1a, 1b, 2a, 2b) and the amino acid sequence derived therefrom (Fig. 3a and 3b). The enzymes of the individual parasites can, however, differ from parasite to parasite. Such variations of the amino acids are usually amino acid exchanges. There can, however, also be deletions, insertions and additions of amino acids to the total sequence. The enzymes according to the invention, both in size and type depending on the cell and cell type in which they are expressed, can be glycosylated or non-glycosylated.

The enzymes according to the invention or fragments of these enzymes are produced by expression of the DNA according to the invention in suitable expression systems, for example in bacteria, in particular in E. coli, as prokaryotic expression system or in a eukaryotic expression system, in particular COS-cells or Dictyostelium discoideum.

With the aid of the nucleic acid sequence according to the invention, it is possible to look for the coding gene or its variants in the genome of any parasite, to identify these and to isolate the desired coding gene for the enzymes. Processes of this type and screening processes suitable for this purpose are known to the person skilled in the art.

As a result of the application of recombinant technology, it is possible to produce a multiplicity of variants of enzymes or fragments of enzymes. Derivatives of this type can be modified, for example, in one or more amino acids by substitution, deletion or addition. The derivation can be, for example, by site directed mutagenesis. Variations of this type can easily be carried out by the person skilled in the art. It merely has to be ensured that the characteristic properties of the enzymes are retained. A further subject of this invention is therefore the enzymes, which are involved in the DOXP metabolic pathway, in particular DOXP-synthase and DOXP-reductoisomerase, which

a) are the product of a prokaryotic or eukaryotic expression of an exogenous DNA,
b) are coded from a sequence in Fig. 1a, 1b, 2a and 2b,
c) are coded from DNA-sequences which hybridise with the DNA-sequences shown in Fig. 1a, 1b, 2a and 2b or fragments of these DNA sequences (see, for example, Fig. 4a and 4b) in the DNA region which codes the mature protein, or

d) are coded from DNA-sequences which would hybridise without degeneration of the genetic code with the sequences defined in b) to c) and code a polypeptide with the same amino acid sequence.

Enzymes are preferred which are coded from the nucleotides from Fig. 1a, 1b, 2a and 2b or from DNA-sequences which, due to the degeneration of the genetic code, would code a polypeptide with the same amino acid sequence.

The two enzymes according to the invention (sequence in Fig. 3a and 3b) can be seen as new prototypes of specific proteins, unicellular and multicellular parasites, in particular of the unicellular parasites.

This invention relates to nucleic acid sequences which code the enzymes and are selected from the group

a) of DNA sequences shown in Fig. 1a, 1b, 2a and 2b or their complementary sequences,
b) nucleic acid sequences which hybridise with one of the sequences in a),
c) nucleic acid sequences which would hybridise with one of the sequences mentioned in a) or b) without degeneration of the genetic code.

The invention also relates to enzymes from any parasites which essentially condense pyruvate and glyceraldehydes-3-phosphate to 1-desoxy-D-xylulose-5-phosphate (DOXP-synthase) and convert 1-desoxy-D-xylulose-5-phosphate to

2-C-methyl-D-erythritol-4-phosphate (DOXP-reductoisomerase). These enzymes, similar to the enzymes from malaria parasites, can be obtained in that a cDNA library or genomic library of the corresponding parasites is screened by processes familiar to the person skilled in the art with a hybridising probe containing enzymes from sequences coding malaria parasites, or by the sequence comparison of the DNA and protein sequence for enzymes of malaria parasites with other parasite enzymes.

With the aid of the nucleic acids, enzymes according to the invention can be obtained in large quantities in a repeatable manner. The nucleic acid is integrated into suitable expression vectors by processes familiar to the person skilled in the art, for expression in prokaryotic and eukaryotic organisms. An expression vector of this type preferably contains an adjustable/inducible promoting agent. These recombinant vectors are then introduced by known processes into suitable host cells for expression and the transformed, transfected or transduced host cells are cultivated under conditions which allow an expression of the heterologous gene. Suitable host cells include prokaryotic cells such as, for example, E. coli, and eukaryotic cells, in particular yeasts (for example Saccharomyces cervisiae, Schizosaccharomyces pombe, Pichia pastoris), insect cells, (for example cell lines of Drosophila melanogaster such as S2 cells, Spodoptera frugiperda, Trichoplusia ni), vertebrate animal cell lines,

particularly teratocarcinoma cell lines such as CHO or COS cells and plant cell lines.

The enzymes according to the invention can also be expressed in transgenic plants and animals, (for example mice, sheep, goats, pigs, guinea pigs). Advantageously the expression system is to be arranged by techniques known to the person skilled in the art, in such a way that the enzymes produced are separated off with the milk of the animals or can be obtained from easily obtained plant parts (fruit, leaves, blossom, shoot and root parts).

Particularly suitable as expression vectors for vertebrate animal cell lines are systems derived from papilloma viruses (for example SV40), retro viruses, sindbis viruses, cytomegalo viruses and vaccinia viruses. Particularly suitable for insect cells is the baculo virus system, for plants, particularly suitable are cell systems based on the ti-plasmid of Agrobacterium tumefaciens and the bombardment of cells with particles covered with nucleic acid.

The expression of the enzymes according to the invention is particularly significant in slime fungi such as Dictyostelium discoideum, Polysphondylium pallidum and Physarum polycephalum as their cells can be cultivated economically in large quantities on simple media. The use of Dictyostelium discoideum offers the further advantage that this organism uses similar codons for the respective amino acids such as Plasmodium falciparum and

particularly effective production of the enzymes according to the invention is thus achieved. Moreover, inducible promoting agents (for example due to lack of food) are known for expression vectors for Dictyostelium discoideum. As a result the recombinant enzyme yield can be further increased.

Particularly suitable for the expression of the enzymes according to the invention are host cells and organisms of the type which have no intrinsic enzymes which condense pyruvate and glyceraldehyde-3-phosphate to 1-desoxy-D-xylulose-5-phosphate (DOXP-synthase) and react 1-desoxy-D-xylulose-5-phosphate to 2-C-methyl-D-erythritol-4-phosphate (DOXP-reductoisomerase). This applies to archaebacteria, animals, fungi, slime fungi and some eubacteria. Detection and purification of the recombinant enzymes is substantially facilitated by the lack of these intrinsic enzyme activities. Moreover, it is possible for the first time to measure economically the activity and in particular the inhibition of the activity of the recombinant enzymes according to the invention by various chemicals and pharmaceutical compositions in raw extracts from the host cells.

The enzymes according to the invention are advantageously expressed in eukaryotic cells, when post-translatory modifications and a native folding of the polypeptide chain is to be achieved. Moreover, as a function of the expression system, during the expression of genomic DNA-sequences, introns are eliminated by splicing the DNA and the enzymes are produced in the

polypeptide sequence characteristic for the parasite. Sequences coding introns can also be eliminated from the DNA-sequences to be expressed by recombinant DNA technology or inserted experimentally.

The protein can be isolated from the host cell or the culture supernatant of the host cell by the process known to the person skilled in the art. An in vitro reactivation of the enzymes may also be required.

To facilitate purification, the enzymes according to the invention or fragments of the enzymes can be expressed as fusion protein with various peptide chains. Oligo-histidine sequences and sequences derived from the glutathione-S-transferase, thioredoxin or calmodulin-binding peptides are particularly suitable for this. Fusions with thioredoxin derived sequences are particularly suitable for prokaryotic expression as the solubility of the recombinant enzymes is thus increased.

Furthermore, the enzymes according to the invention or part sequences of the enzymes can be expressed with peptide chains, known to the person skilled in the art, which are such that the recombinant enzymes are transported into the extra cellular milieu or into certain compartments of the host cells. As a result, purification and investigation of the biological activity of the enzymes can be facilitated.

With the expression of the enzymes according to the invention, it may prove expedient to change individual

codons. Specific exchange of bases in the coding region is also sensible when the codons used in the parasites are different from the codons used in the heterologous expression system to ensure optimum synthesis of the protein. Deletions of non-translated 5′ or 3′ portions are also often sensible, for example when a plurality of destabilising sequence motives ATTTA are present in the 3′ region of the DNA. These should then be deleted in the preferred expression in eukaryons. Changes of this kind are deletions, additions or exchange of bases and also subject of the present invention.

The enzymes according to the invention can further be obtained under standardised conditions by techniques known to the person skilled in the art by in vitro translation. Systems which are suitable for this are rabbit reticulocytes and wheat germ extracts. Also, mRNA transcribed in vitro can be translated into xenopus-oocytes.

By virtue of chemical synthesis, oligo- and polypeptides can be produced, the sequences of which are derived from the peptide sequence of the enzymes according to the invention. With suitable choice of the sequences, peptides of this type have features which are characteristic of the complete enzymes according to the invention. Peptides of this type can be produced in large quantities and are particularly suitable for studies on the kinetics of enzyme activity, the adjustment of enzyme activity, the three-dimensional structure of enzymes, the inhibition of enzyme activity

by various chemicals and pharmaceutical compositions and the binding geometry and binding affinity of various ligands.

A DNA with the nucleotides from the sequences shown in Fig. 1a, 1b, 2a and 2b or a fragment according to Fig. 4a and 4b is preferably used for the recombinant production of enzymes according to the invention.

The invention also relates to processes for obtaining enzymes involved in the DOXP metabolic pathway, in particular the enzymes DOXP-synthase and DOXP-reductoisomerase by isolating from the parasites. The enzymes are isolated from parasite extracts by chromatographic, electophoretic and other processes known to the person skilled in the art. The enzymes are found by measuring the respective enzymatic activity or reactivity with appropriate antibodies.

The detection of transformed, transfected or transduced host cells which recombinantly produce the enzymes and the purification of the protein are preferably by antibodies which bind to these enzymes. Antibodies of this type can be obtained easily with the aid of the enzymes according to the invention or parts of the enzymes as antigen or immunogen by known processes.

Homologous or cross-converting proteins of other parasites can be detected with the antibodies to proteins according to the invention, for example, by the Western blot test.

This invention also relates to methods for determining the enzymatic activity of the DOXP enzymes, in particular of the enzymes DOXP-synthase and DOXP-reductoisomerase. This can be determined according to known instructions (Sprenger et al. PNAS, 94 (1997) 12857-62 und Kuzuyama et al. Tetrahedron Letters 39 (1998) 4509-12). In this process the condensation of pyruvate and glyceraldehyde-3-phosphate to 1-desoxy-D-xylulose-5-phosphate (DOXP-synthase) and the conversion of 1-desoxy-D-xylulose-5-phosphate to 2-C-methyl-D-erythritol-4-phospate (DOXP-reductoisomerase) is detected. This invention also relates to the use of these measuring processes for obtaining substances which inhibit the activity of the respected enzymes.

By the application of recombinant technology it is possible to produce a multiplicity of variations of enzymes or fragments of enzymes. Derivatives of this type can, for example, be modified in one or more amino acids by substitution, deletion or addition. The derivation can, for example, be by site directed mutagenesis. Variations of this type can easily be carried out by the person skilled in the art. It must merely be ensured that the characteristic features of the enzymes are retained.

With the help of the enzymes according to the invention and their homologues, new specific active ingredients against parasites can be found.

In particular, the detection processes described above can be used in appropriate test kits for screening for anti-parasitic activity of substances. These include processes known to the person skilled in the art and suitable for screening natural substances from flora and fauna, from plants, algae, bacteria or animals, and their derivatives, chemical libraries, also libraries which have been compiled by means of techniques known to the person skilled in the art, including combinatory chemistry. (Pindur et al. Pharmazie in unserer Zeit 26 (1997) 24-30; Broach et al. Nature 384 (1997) 14-6; Lack et al. Chimia 50 (1996) 445-7; Czarnik und Ellmann Accounts of Chemical Research 29 (1996); Chemical and Engineering News 74 (1996) 28-73; Lorin et al. Chemical Reviews 96 (1996) 555-600; Weber et al. Nachrichten aus Chemie, Technik und Laboratorium 42 (1994) 698-702).

The present invention also relates to the use of proteins or fragments of these proteins, including proteins or fragments of proteins with or without enzymatic activity in techniques known to the person skilled in the art for determining structures of protein, in particular the characterisation of binding sites suitable for the development of preparations with inhibiting effect on enzymatic activity.

Active ingredients obtained with the aid of proteins according to the invention are of great interest to medicine and veterinary medicine.

Active ingredients found with the aid of the proteins according to the invention are suitable, in favourable homoeothermic toxicity, for fighting pathogenic parasites which occur in humans and in animal husbandry and rearing in domestic, breeding, zoo, laboratory and animals for experimentation and pets. They are effective here against all or individual stages of development of the destructive parasites and against resistant and normally sensitive parasites. As a result of the fight against parasites, diseases, fatalities and reductions in performance (for example in the production of meat, milk, wool, skins, eggs, etc) should be reduced, so the use of active ingredients allows easier and more cost-efficient animal husbandry.

By using these processes according to the invention including established assays, it could be shown that the activity of the DOXP-reductoisomerase is inhibited by 3-(N-acetyl-N-hydroxyamino)propylphosphonate and derivatives 3-(N-formyl-N-hydroxyamino)propylphosphonate (fosmidomycin). Both substances originate from a chemical library of acylhydroxylamino-alkylphosphonic acid derivatives. This group of compounds was described in the past as herbicidal and bactericidal (US 4693742, DE2733658). The efficiency of the system for obtaining anti-parasitic active ingredients has been shown here. The results from the enzyme assays could be confirmed both in the malaria culture (see examples) and in the animal experiment (see examples). The inhibitors found by means of these enzyme assays were able to inhibit the

growth of malaria parasites in vitro and in vivo. Treatment of animals over a time period of 8 days showed a healing of the animals. The acetyl form showed a three times greater efficacy than the formyl form. This result is very surprising, as substantially higher (up to 1000x) concentrations of 3-(N-acetyl-N-hydroxyamino)propylphosphonate were needed to inhibit the bacteria growth.

The process according to the invention is thus suitable for identifying active ingredients and the active ingredients according to the invention are suitable for the therapeutic and prophylactic treatment of infections in humans and animals caused by parasites, fungi or viruses. The compounds are suitable as prophylactics against, and for treatment of, infections, caused by pathogens of malaria and sleeping sickness as well as Chagas' disease, toxoplasmosis, amoebic dysentery, leishmanosis, trichomoniasis, pneumocystosis, balantidiosis, cryptosporidiosis, sarcocystosis, acanthamebiasis, naegleriasis, coccidiosis, giardiasis and lambliosis.

The processes according to the invention and the active ingredients according to the invention are particularly suitable for treating malaria, sleeping sickness and leishmanoses.

The active ingredients according to the invention are also suitable for inhibiting the metabolic pathway of bacteria and plants. Substances which are identified

according to the invention as inhibitors of DOXP metabolic pathway are therefore also suitable for use as herbicides and for use in treating bacterial infections in humans and animals.

Domestic and breeding animals suitable for treatment include mammals such as, for example, cattle, horses, sheep, pigs, goats, camels, water buffalos, donkeys, rabbits, salt and freshwater fish as, for example, trout, carp and eels. Suitable laboratory animals and animals for experimentation include mice, rats, guinea pigs, golden hamsters, dogs, cats and pigs. Suitable pets include dogs and cats. Application can be both prophylactic and therapeutic. The application of active ingredients is direct or in the form of suitable preparations known to the person skilled in the art such as enteral, parenteral, dermal or nasal.

The active ingredients according to the invention can be used in combination with any anti-infective agents known to the person skilled in the art. These include substances which have an antibacterial, antiparasitic, antiviral or fungicidal effect. These include anti-infective agents which are listed in the red list and in the specialist literature (Allegemeine und spezielle Pharmakologie und Toxikololgie von Forth et al. BI-Wissenschaftsverlag, Mannheim 1998; Antibiotikatherapie von Simon und Stille, Schattauer-Verlag, Stuttgart 1993).

As some parasites have both the mevalonate metabolic pathway and the DOXP metabolic pathway, the invention also relates to the combination of inhibitors of the DOXP metabolic pathway with preparations which inhibit the fat metabolic pathway, including inhibitors of the synthesis or absorption of lipids, in particular, inhibitors of the mevalonate metabolic pathway. The inhibitors of the enzymes HMG-CoA-synthase and inhibitors of the HMG-CoA-reductase deserve particular mention. Included amongst the inhibitors of the HMG-CoA-reductase are, in particular, Lovastatin and derivatives, Mevastatin and derivatives, Compactin and derivatives, Simvastatin and derivatives, Pravastatin and derivatives, Atorvastatin and derivatives, Fluvastatin and derivatives and Cerivastatin and derivatives.

Example 1

Expression cloning of the gene of P. falciparum coding the DOXP-reductoisomerase.

The gene coding the DOX-reductoisomerase of P. falciparum was cloned by PCR amplification of the corresponding sequences of genomic DNA as matrix. To obtain genome DNA, the P. falciparum strain HB3 was cultivated by the Kerzentopf process (Tranger und Jensen (1976), Science 193, 673-675). As culture medium, RPMI 1640 (with HEPES and L-glutamine, Gibco) was supplemented with 10% of human serum, 0.3 µg/ml of Gentamicin and 0.1 mM of Hypoxanthine and a hematocrit

of 5% adjusted with human erythrocytes. 15 culture dishes with 35 ml culture volume in each case were used with approximately 4% of parasitemia for the preparation of the DNA. The infected erythrocytes were harvested by centrifugation and washed twice in carrier buffer (57 mM NaCl, 58 mM KCl, 1 mM NaH_2PO_4, 7 mM K_2HPO_4, 11 mM $NaHCO_3$, 14 mM glucose). The parasites were released from the erythrocytes by lysing the cell sediment with a ten-fold volume of 1% saponin solution in carrier buffer for 5 minutes on ice (modified according to Kilejian (1979), Proc. Natl. Acad. Sci. USA 76, 4650-4653). The free parasites were washed twice by centrifugation (10 min, 10,000 rpm, 4°C) with a solution of 1% BSA in carrier buffer. The DNA preparation from the free parasites obtained took place according to standard procedures. The parasites were then digested with proteinase K. The assay was then extracted four times with phenol/chloroform, the DNA solution was dialysed overnight against TE and then precipitated with isopropanol. The following primer was used for the PCR amplification:

PfYAEMfor 5`-CTGAATTTCATATTACAAAATTAATAGATG-3`
PfYAEMrev 5`-GTACTATGAAGAATTATGTTTGTTGTATAT-3`

The following assay was used for the PCR conversion:

3 µl 10 x PCR-buffer
2.4 µl 25 mM $MgSO_4$
2.4 µl 2.5 mM dNTP

2 µl DNA matrices (0,2 µg/ml)
2 µl primer 1 (7.5 µM)
2 µl primer 2 (7.5 µM)
0.2 µl tag-polymerase (5 U/µl)
16 µl H_2O

The amplification took place with the following profile:

3 cycles:	96°C	1 min
	48°C	1 min
	72°C	3 min
32 cycles:	95°C	40 sec
	48°C	1 min
	72°C	3 min

After the last cycle the assay was incubated for a further 10 minutes at 72°C to lengthen all the products. The PCR product of four assays of this type were combined and purified with a 0.7% agarose gel. The elution of the DNA from the agarose blocks took place with the "kit for DNA extraction" (Millipore, Cat. No. S667). The eluted DNA was precipitated with ethanol and absorbed in 10 µl H_2O. The PCR product was then cloned according to the manufacturer's instructions with the TA cloning kit (in vitro gene). 20 mg of insert-DNA were used for a ligation assay. Colonies of bacteria bearing the desired recombinant plasmid were identified by analytical plasmid preparation and EcoR I-digestion of the plasmids. The cloned PCR products were then sequenced using standard, forward and reverse primers; the sequences were completed with the Walkings primer technique.

A PCR product, present in the corresponding orientation in the pCR2.1 vector, was re-cloned in the expression vector pBK-CMV (Stratagene) for expression in COS-7-cells. The re-cloning took place via the intersections of the restriction enzymes Not I and BamH I, which occur in the polylinker of the two vectors. For the transfection of the COS-7-cells, the expression vector with the PCR product as insert was produced on a preparative scale by anion exchange chromatography (Qiagen).

All the methods used for the cloning are described in detail in J. Sambrook, E.F. Fritsch, T. Maniatis (1989), Molecular cloning: a laboratory manual, 2nd edition, Cold Spring Habor Laboratory Press, Cold Spring Habor, USA.

The COS-7-cells were cultivated in DMEM medium with 10% FCS under standard conditions. 30 ml of culture medium were allowed per cell culture bottle. Cells with approximately 50% confluency were used for the transfection, which had been split the day before. DOTAP (Boehringer) was used as transfection reagent. 40 µl of DNA solution (0.5 µg/ml) were mixed with 110 µl of 20 mM HEPES (pH 7.4). 100 µl of DOTAP were also mixed with 230 µl 20 mM HEPES (pH 7.4) in a polystyrene conversion vessel. Then the DNA solution was pipetted into the DOTP solution and incubated for 15 minutes at room temperature. Then the assay was mixed with 20 ml of culture medium and the medium of the COS-7-cells was replaced by this mixture. The next day the cells were

transferred with fresh medium into new cell culture bottles. After a further 48 hour incubation the transfected COS-7-cells were harvested. The cells were scraped off for this purpose and washed 3 times by centrifugation in assay buffer (100 mM TrisHCl (pH 7.5), 1 mM $MnCl_2$). The cells were suspended again in a minimal volume of assay buffer and digested by being frozen three times (in liquid nitrogen) and thawing. Cell fragments were centrifuged off in a 1.5 ml conversion vessel (13,000 rpm, 10 min, 4°C) and the supernatant used directly for measuring the enzyme activity or purifying the enzyme.

Example 2

Purification of the recombinant DOXP-reductoisomerase of P. falciparum.

The recombinant DOXP-reductoisomerase of P. falciparum expressed in COS-7-cells was purified for considerable homogeneity and more precise characterisation. The purification took place in an affinity chromatography and a gel permeation chromatography step. Antibodies against the DOXP-reductoisomerase of P. falciparum were produced for the production of a suitable affinity chromatography column. Portions were also chosen from the amino acid sequence derived from the DNA sequence, for which a particularly high antigen effect could be predicted. Appropriate peptides were synthesised and used for immunising rabbits. The quality of the antisera obtained was confirmed by its reactivity with the

synthetic peptides and by Western blot tests. For the Western blot tests (BM Western Blotting Kit, Boehringer) extracts were used from the P. falciparum and recombinant COS-cells.

The antiserum for eliminating low-molecular amines was dialysed against PBS for producing the affinity chromatography column. The antibodies were bound to protein A-sepharose and covalently coupled by cross-linking with DMP (IgG Orientation Kit, Pierce). The protein extract was, as described in Example 1, obtained from 55 cell culture bottles with transfected COS-7-cells and loaded on to the columns equilibrated with assay buffer. After excessive washing with assay buffer the column was eluted with elution buffer (100 mM GlycinHCl (pH 2.8) 0.4% CHAPS). The eluate was immediately neutralised with 1 M TrisHC1 (pH 7.5). The main fractions were identified by Western blot analysis. Biotinylated antibodies were used for detection to avoid disruption by antibodies eluted from the column in small quantities. The main fractions were combined, dialysed against assay buffer and concentrated by ultrafiltration (30 Kda, Amicon). Further purification took place by gel permeation chromatography (Superdex 200, Pharmacia) with assay buffer as input and elution buffer. The main fractions were identified as described above, combined and concentrated, reacted with 20% glycerol and frozen at -70°C. As a result of SDS-PAGE (12% acrylamide) under reducing conditions and silver staining (Gel Code Silver Stain Kit, Pierce), the cleaned DOXP-reductoisomerase of P. falciparum was shown as a unified band at 54 kDa.

Example 3

Determination of the activity of the purified enzyme and screening for inhibitors.

The DOXP-reductoisomerase activity of the purified enzyme was confirmed in an in vitro test system. 100 µl of assay buffer with 0.3 mM NADPH, 0.3 mM DOXP and 10 µg of recombinant enzyme were used for a typical test assay. The conversion was started by the addition of DOXP to the complete assay. The oxidation of NADPH was carried out photometrically with 340 nm in microquartz cuvettes at 37°C. This test system was used to show the inhibition of recombinant DOXP-reductoisomerase of P. falciparum by various substances. After addition of 1 µM 3-(N-formyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt and 1 µM 3-(N-acetyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt) to the conversion assay no change was observed to the absorption at 340 nm. Under these conditions, the DOXP-reductoisomerase of P. falciparum was completely inhibited.

Example 4

Test of the effectiveness of the substances against malaria in vivo.

The various derivatives were tested by the modified Peters' test. The substances were applied in a quarter of the median lethal dose (LD50). In the test assay, 10 mice were infected with Plasmodium vinckeii, the pathogen of mouse malaria. Once infection had been confirmed by blood examination, the 4 mice were treated. 6 mice which had not been treated were used as controls. The treatment with 1-1000 mg/kg/d 3-(N-formyl-N-hydroxylamino)-propyl phosphonic acid monosodium salt over 3 days led to a destruction of the parasites in the blood of the mice. The treated group was free of living parasites after only 1 day. The control mice had to be destroyed on day 5 after infection with a parasitemia of > 80%. The treated mice were still free of parasites 8 weeks after the end of the treatment. Further experiments showed an effectiveness of 50 mg/kg/d 3-(N-formyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt in mice with a parasitemia of 80%. These mice were also free of living parasites after 1 day. Further results for 3-(N-formyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt and 3-(N-acetyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt are shown in Fig. 5.

Example 5

Protection from malaria in the experiment with infected mice.

The effectiveness of the compounds in vivo against malaria was tested by using male mice (BALB/c-strain)

weighing 20 to 25 g. One day before the infection, 4 mice were treated intraperitoneally with 50 mg/kg of 3-(N-formyl-N-hydroxylamino)-propyl-phosphonic acid monosodium salt. The mice were then infected with Plasmodium vinckeii. Mice which had not been pre-treated with the substance were used as controls. No infection could be detected in the treated mice, while the control mice were destroyed after 5 days with a parasitemia over 80%. The treated mice were free of parasites 8 weeks after the infection.

Example 6

In vitro inhibition of the growth of malaria parasites on the principle of the IC50 determination (the concentration at which the vitality of the parasites is reduced by half).

For determining the IC50 values, the malaria parasites are initially cultivated for a complete 48 hour cycle in the presence of inhibitors, in the next 24 hours the survival rate was measured by [^{3}H]-hypoxanthine insertion. A dilution series of 3-(N-formyl-N-hydroxylamino)-propyl-phosphonic acid mono-sodium salt was presented on a micro-titre plate in 20-μl-aliquots concentrated by 10. Then 180 μl of parasite suspension in culture medium was added to each well. Asynchronous cultures with about 0.4% parasitemia and 2% hematocrit were used. The micro-titre plates were then incubated for 48 hours. Then 30 μl [^{3}H]-hypoxanthine were added to

each well. After incubating for 24 hours the cells were harvested and the incorporated radioactivity was measured. The results with the HB3, A2 and Dd2 strains with known resistances against other malaria medications is shown in Fig. 6a, 6b and 6c. In both strains, an IC-50 value of below 0.5 μM occurs. The resistances of these strains are:

Plasmodium falciparum HB3 (Honduras) is resistant to Pyrimethamine.

Plasmodium falciparum Dd2 (Indochina) is resistant to chloroquine, quinine, pyrimethamine, cycloguanil and sulfadoxine.

Plasmodium falciparum A2 (Gambia) is resistant to chloroquine and cycloguanil.

No cross resistances were found with anti-malaria preparations.